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Partial Repression of Human γ-Globin Genes by LCR Element HS3 When Linked to β-Globin Genes and LCR Element HS2 in MEL Cells

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Clues for overcoming fetal (γ-) globin gene repression in adult human erythroid cells may come from understanding why repression of isolated γ-globin genes has not previously been achieved in the adult erythroid environment of mouse erythroleukemia cells (MEL). Repression of human γ-globin genes has been demonstrated in MEL cells when transferred as part of the entire β-globin gene cluster packaged in chromatin. Major differences in these approaches are prior packaging into chromatin and the presence of additional sequences, notably from the locus control region (LCR). In this report we focus on the contribution to γ-globin gene repression that multiple elements of the LCR may have. We first show preferential activation of β-globin genes over γ-globin genes in MEL cells when linked to each other and to LCR sequences containing the core elements of DNase I hypersensitive sites 4, 3, and 2. Removal of the HS4 element had no effect, however, removal of the 225 bp HS3 core element resulted in a five-fold increase in γ-globin gene expression. The enhancer 3′ to the Aγ-globin gene also had no apparent effect on γ-globin gene expression. These results provide first evidence of γ-globin gene repression involving the core region of HS3 in the presence of the core region of HS2 and a β-globin gene. A mechanism for repression involving sequestration of the γ-promoter away from the strong enhancer activity of HS2 is proposed.

Key words: LCR, β-globin gene, γ-globin gene, MEL

INTRODUCTION

Repression of human fetal globin gene expression is an intrinsic property of adult erythroid progenitors [1]. The degree of fetal globin gene repression can be modulated both early and late during erythropoiesis by environmental factors such as pharmacologic agents [2–4]. Understanding the molecular basis for modulating fetal globin gene repression during erythropoiesis may be important for treating sickle cell disease and thalassemia with drug therapy.

Transgenic mice have proven useful in replicating the developmental switch from human fetal to adult globins. Although mice do not have a fetal-stage globin, transgenic mice are able to correctly repress expression of human fetal (γ) globin genes in adult cells [5–7]. In general, it has been found that including more β-globin gene cluster sequence in the tested construct results in more faithful γ to β switching in transgenic mice. Reduction of sequence to the individual genes and the HS2 element from the locus control region (LCR) still retains some switching but the HbF levels are generally higher than the 1% or less found in normal human adults [8–10]. By contrast, constructs with the HS2 element and individual globin genes or the whole β-globin cluster in mouse erythroleukemia (MEL) cells express γ- and β-globin genes equally well [11]. Repression of γ-globin genes in MEL cells has been demonstrated but only in chromosome transfer experiments when the γ-globin genes were transferred as part of an entire β-globin gene cluster [12–14] and not as individual genes [15]. These findings indicate that complete γ- to β-globin gene switching requires appropriate packaging of the globin genes achieved in transgenic mice and in the chromosome transfer studies. The findings also suggest that sequences beyond isolated globin genes and HS2 are required.

Candidate sequences tested in this study were addi-

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tional core elements from the LCR and the enhancer 3′ to the Aγ-globin gene. The LCR consists of four highly hypersensitive DNase I sites and are thought to establish active chromatin domains in erythroid cells [16]. HS2 is the only site with classic enhancer activity in transient expression studies while HS1 is minimally active. The core activities of sites HS4, 3 and 2 have recently been mapped, and developmental stage-specific enhancement of linked γ- and β-globin genes by individual HS elements in transgenic mice is associated with HS4 and HS3 but not HS2 [17]. The core elements retain some but not all of the activity of the full LCR. This disadvantage is countered by the ability to use the core elements to focus on regulatory properties of highly localized areas. The small size of core elements also makes them attractive for use in viral vectors for gene therapy [18,19]. The region 3′ to the Aγ-globin gene was originally identified as having enhancer activity in K562 cells, a human myelomonocytic line with embryonic/fetal erythroid properties [20]. Subsequent studies have implicated this region as repressing γ-globin expression in adult cells [9,21]. An enhancer 3′ to the β-globin gene has also been described which is active in adult transgenic mice [22,23].

Our hypothesis was that repression arising from chromatin packaging and repression arising from the presence of regulatory sequences are separable and could be studied independently. In this vein, we have used MEL cells to test function of specific regions of the LCR on the expression patterns of γ- and β-globin genes. Our goal was to determine whether certain sequences in the LCR would preferentially activate β- over γ-globin expression. Any repression resulting from inclusion of LCR sequences (or the enhancers) would argue for the involvement of those sequences in actively down regulating γ-globin expression. Additional sequences or modes of repression may be required for complete repression and will be the subject of further investigation in systems like transgenic mice.

We included HS4, HS3, the 3′ Aγ enhancer, and the 3′ β enhancer in our constructs to determine whether these sequences would contribute to γ-globin gene repression in the adult erythroid environment of MEL cells. The combinatorial action of LCR elements on individual globin genes [24–26] and the action of individual LCR elements on both γ- and β-globin genes [8,9,10,17] have been studied in MEL and transgenic mice. Here we present a first examination of the combinatorial action of LCR elements on both γ- and β-globin genes.

MATERIALS AND METHODS

Constructs

Core LCR fragments were prepared from plasmids p5′ε7, p5′ε6, and p5′ε3 (kindly provided by Dr. P. Powers) containing HS4, HS3, and HS2, respectively (Fig. 1A) [27]. The 0.3 kb SacI-DraI fragment containing the core activity of HS4 was cloned into the pUC19 polylinker SacI and SmaI sites. The 0.25 kb Hph I-Fnu4H I fragment containing the core activity of HS3 was PCR amplified using the primers;

HS3 5′ (−15,008): 5′TACCCGGGTCACGGTGACTTTGCGA3′

HS3 3′ (−14765): 5′TAGGATCCGCTGCTATGCTGTGCCT3′

and cloned into the polylinker Sma I and BamH I sites. For HS2, the 0.5 kb Hae III fragment was first PCR amplified using primers;

HS2 5′ (−10975): 5′ATAGATCTGGCCAGGCCCCTGTCGG3′

HS2 3′ (−10538): 5′CGCTCGAGGCCACCTGCAAGATAAA3′.

The 0.3 kb Bgl II-Xba I region from the 5′ region of the HS2 PCR product was cloned into the BamH I-Xba I polylinker sites. Cloned HS3 and HS2 sites were sequenced to check for mutations introduced by PCR, and none were found. Plasmids p432, p32, and p2 refer to which HS sites are present in the construct.

The γ-globin gene used was the 2.1 kb Stu I-Sph I fragment (−390 to +1,700 from the cap site, Fig. 1A) from $^{G/A}$γ-SVX cloned into the Hinc II and Sph I sites of the pUC19 polylinker of p432 to give p432γ [28]. The γ-globin gene was modified to contain the 0.2 kb Acc I-BamH I fragment from the β-globin second exon for future use in human cells. Additionally, the γ-globin gene was cloned into p32 and p2 to give p32γ and p2γ.

The 3′ Aγ enhancer (Fig. 1A, kindly provided by R. Emery, CHOP) used was the 0.8 kb Hind III fragment amplified from the cosmid clone HG28 and checked by sequencing that no PCR-induced mutations were present. The Aγ enhancer was cloned into the Hind III site of p432γ in the same orientation as the γ-globin gene to give p432γE′.

The β-globin gene used was the 2.4 kb SnaB I-Hpa II fragment (Fig. 1A, −256 to +2,150 from the cap site) cloned into the Hinc II site of the pUC19 polylinker after filling in the 5′ overhang of Hpa II with Klenow fragment DNA polymerase. The second exon Acc I-BamH I fragment was replaced with the homologous fragment from the γ-globin gene. The β-globin gene was cloned into p432 to give p432β.

The 3′ β enhancer used was the 0.25 kb Pst I fragment (Fig. 1A) cloned into the Pst I site 3′ to the inserted β-globin gene of p432β to give p432βE. Clones used contained the enhancer and β-globin gene in the same orientation as determined by sequencing.

To prepare p432γβ and p432γβE, the β and βE fragments were excised using flanking sites in the polylinker as Xba I-Hind III fragments and cloned in the same orientation as γ into the Hind III site of p432γ by blunt end ligation after blunting all overhanging sites. p32γβE was prepared in the same way. The same approach was

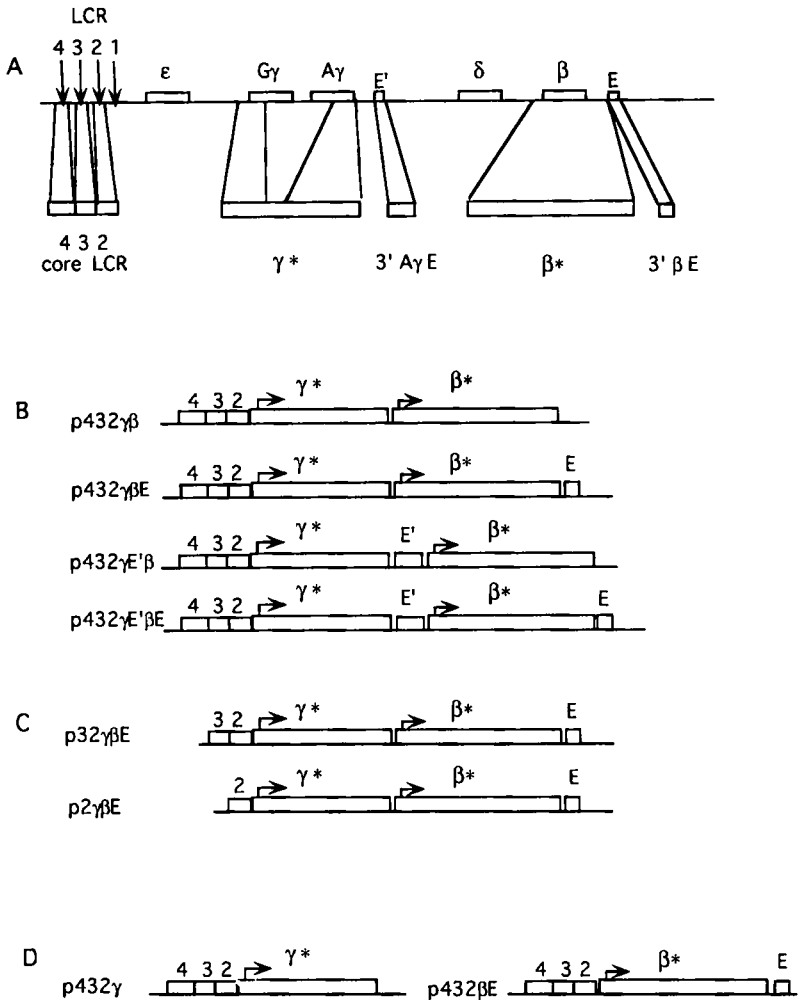

Fig. 1. Globin gene constructs used in this study. See text for details of construction. A: Schematic of the human β-globin gene cluster. Sequences used in preparing the constructs are indicated. The asterisk denotes modification of the second exon of both γ- and β-globin genes for future experiments in human cells as described in the methods section. B: Linked constructs with the core LCR consisting of HS4, HS3, and HS2. C: Linked constructs with HS3 and HS2 or HS2 alone. D: Unlinked constructs with the core LCR consisting of HS4, HS3, and HS2.

used to prepare p432γE′β and p432γE′βE; however, p432γE′ was partially digested with Hind III and clones isolated which contained the β-globin gene (and β enhancer) inserted 3′ to Aγ enhancer and in the same orientation as the γ-globin gene.

Finally, p2γβE was prepared by replacing the Nde I-Xho I fragment of p432γβE with the homologous fragment from p2γ. The exchanged fragment contains the LacZ gene from pUC19, the LCR and the 5′ end of the γ-globin gene.

Cell Culture and Transfection

The MEL cell line 745 (GM00086E, Coriell Institute for Medical Research, Camden, NJ) was maintained at 37°C and 5% CO_2 in Dulbeccos Modified Essential Medium supplemented with 10% (v/v) bovine calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

Globin constructs were cotransfected with pSV2neo into MEL cells using the lipofection agent, DOTAP (Boehringer-Mannheim, Indianapolis, Indiana) according to manufacturer's instructions. Ten micrograms of Qiagen purified plasmid with linked globin genes (5 μg each of plasmids with unlinked globin genes) and 1 μg of pSV2neo were added to 250 μl of HBS (150 mM NaCl, 20 mM Hepes pH 7.4) and then mixed with a solution of 70 μl of DOTAP diluted to 250 μl of HBS. The DNA-DOTAP solution was incubated for 10 min at room temperature before addition of MEL culture medium up to 14 ml. MEL cells (5×10^5) were then added to DNA-DOTAP mixture and incubated overnight (approx. 16 hr) in 5% CO_2 and 37°C. The cells were then removed and

resuspended in 10 ml of fresh medium. After 2 days, 1 ml of the transfected cell culture was expanded to 5 ml of culture medium containing 0.4 mg/ml G418 (active weight). The remaining 9 ml were induced with 5 mM HMBA for 2 days after which the cells were harvested for transient analysis of globin mRNAs. The cells under G418 selection were continuously grown in the presence of 0.4 mg/ml G418 to isolate pools of stably-transfected cells. About 1×10^6 cells were induced with HMBA for 4 days before harvesting for RNA analysis; DNA was isolated from uninduced cells.

RNA and DNA Analyses

RNA and DNA were isolated from cells as previously described [29]. Globin mRNAs were quantitated using an RNase protection assay. The probe pG1β* was used for simultaneous assay of transfected human and endogenous mouse β-globin genes as previously described [2]. pG1β* contains genomic β*-globin sequences from the promoter Sna B1 site to the second exon Bam HI site joined to β*-globin cDNA sequence from the second exon Bam HI site to the third exon EcoRI site where β* refers to the modified β-globin gene containing the γ-globin second exon. Protected fragments are 143 and 278 nucleotides (nt) for the modified human β-globin mRNA, 143 and 106 nt for reticulocyte human β-globin mRNA, and 120 nt for mouse β-globin mRNA. The probe pG1β was used for simultaneous analysis of modified human γ- and β-globin mRNA to give a 209 nt protected fragment for γ-globin mRNA and a 143 nt protected fragment for β-globin mRNA. The pG1β probe contains genomic β-globin sequences from the Sna B1 site in the promoter (−265 to the cap site) to the Bam HI site at the 3′ end of the second exon. Normal human β-globin mRNA present in reticulocyte RNA gives protected fragments of 209 and 143 nt and was used as a control for determining 1:1 stoichiometry of the γ to β protected fragments. The pG1β probe was used identically to the pG1β* probe except that 6.3 μg/ml RNase A and 1.9 μg/ml RNase T1 were used for digestion. For transient assays, 5 μg of RNA was used while 0.5 μg of RNA was used for assays of stably-integrated lines. Protected fragments from either probe were separated on a 5% (w/v) denaturing acrylamide gel followed by autoradiography. Bands were quantitated using a BioRad 620 densitometer to scan autoradiograms. Gels were directly quantitated with a Molecular Dynamics PhosphorImager for analysis of some of the 32γβE and 2γβE constructs.

The copy number of transfected globin genes relative to endogeous mouse genes was determined using a multiplex PCR approach. A single PCR reaction (50 μl covered by 30 μl of mineral oil) consisted of 100 ng of genomic DNA, 2 mM $MgCl_2$, 50 mM KCL, 10 mM Tris-HCl, pH 8.3, 200 μM each dNTP, 0.5–1.0 μCi of ^{32}Pα-dATP (3,000 Ci/mmol), 1 U Taq Polymerase (PEC), and 0.4 μM of each of the following primers:

human γ-globin 5′ (+58): 5′GTCATTTCACAGAGGAGGAC3′

human γ-globin 3′ (+517): 5′CAGTGCTGAAACATCTCCTG3′

human β-globin 5′ (+257): 5′ATTTTCCCACCCTTAGGCTG3′

human β-globin 3′ (+1245): 5′TGGTAGCTGGATTGTAGCTG3′

mouse β^{maj}-globin 5′ (+60): 5′CACCTGACTGATGCTGAGAA3′

mouse β^{maj}-globin 3′ (+406): 5′CAAGTGATTCAGGCCATCGT3′.

PCR reactions were performed in Coy Tempcycler for 20 to 22 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. Samples were electrophoresed on a native 4% (w/v) acrylamide gel, and autoradiograms of the gel were scanned using a BioRad 620 densitometer for quantitation. Gels were directly quantitated with a Molecular Dynamics PhosphorImager for analysis of some of the 32γβE and 2γβE constructs. As a quantitation control, 1 pg and 10 pg of linearized p432γβE were added to 100 ng of untransfected MEL genomic DNA and used to represent 5 and 50 copies per cell. The ratio of PCR products for transferred β-, γ-, and mouse β-globin for a given sample did not vary much between 20 to 22 cycles of amplification and were therefore considered to reflect linear amplification of the targeted genes. Representative samples were also subjected to standard Southern blot analysis and similar copy numbers were obtained (data not shown). The average of two independent PCR reactions at different cycles were used to derive each data point. The mean γ to β gene copy ratio for stably-transfected MEL cells was 0.70 (SD = 0.37, n = 47) for linked constructs. MEL pools made with linked construct which did not have γ to β gene copy ratios within one standard deviation of the mean ratio were considered suspect for rearrangement and not included in our analyses.

RESULTS

Preferential Expression of a β-Globin Over a γ-Globin Gene

A plasmid containing a core LCR, a γ-globin gene, a β-globin gene, and the β-globin enhancer was prepared (p432γβE, see Fig. 1B) to determine whether preferential expression of the β-globin gene could be observed when the plasmid was transfected into MEL cells. MEL cells express endogenous adult β-globin genes, and therefore, might contain transcription factors which would favor expression of the transfected adult β-globin gene over the transfected fetal stage γ-globin gene. The core LCR used consists of 200–300 bp fragments associated with HS 4, 3, and 2 and shown to contain the core activity of

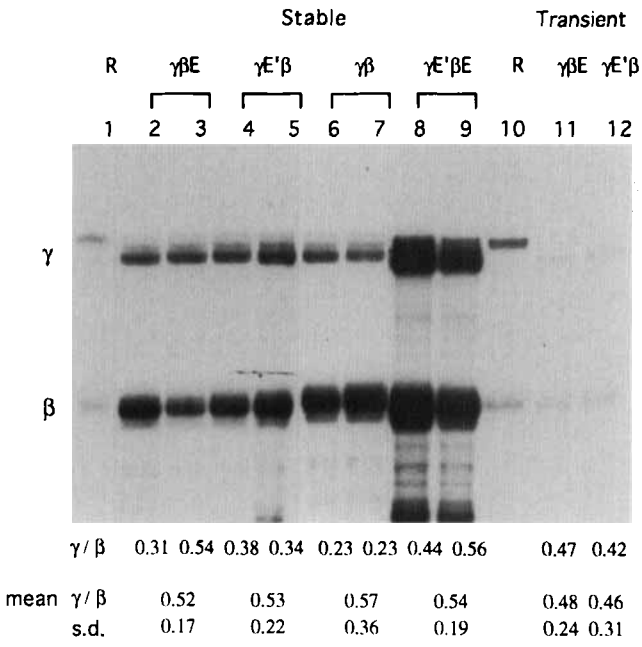

Fig. 2. The γ to β mRNA ratios from linked γ- and β-globin constructs indicate preferential expression of the β-globin gene in MEL cells. Shown are results from an RNase protection assay of two independent stably-transfected pools of MEL cells representative for each construct (lanes 2 to 9). Two representative transiently-transfected pools are also shown (lanes 11 and 12). R, reticulocyte control. The γ to β mRNA ratio for each representative sample shown is given below. Also given are the mean γ to β ratios for pools analyzed [n = 8 except for γβ (n = 5) and γE'βE (n = 6)]. Note that γ-globin gene expression is lower than β-globin gene expression from all constructs containing a LCR with HS4, HS3, and HS2.

these sites in transgenic mice [24]. The γ-globin gene promoter was placed closer to the LCR than the β-globin promoter to reflect the native state.

Expression of the globin genes linked together on p432γβE was analyzed in both stably-transfected and transiently-transfected MEL cells. The ratio of γ to β mRNA for p432γβE was 0.52 (SD = 0.17, n = 8) and 0.48 (SD = 0.24, n = 8) in stably and transient-transfected MEL cells, respectively (Fig. 2, lanes 2, 3, and 11). These results indicate two-fold preferential expression of the β- over the γ-globin gene which is independent of chromosomal integration.

Three constructs similar to p432γβE were prepared which contained the 3′ Aγ enhancer (E′) in addition to the β enhancer (E), just the 3′ Aγ enhancer, or neither enhancer (Fig. 1B). These constructs were used to test whether these enhancers had any effect on the preferential expression of the β-globin gene. The γ to β ratios for p432γE′β, p432γβE, and p432γE′βE in both stably and transient transfected MEL cells were all approximately 0.5 and not significantly different from the ratios obtained with p432γβE (Fig. 2). Thus, in this system γ expression was less than β expression and no effect of the 3′ Aγ or β enhancers on regulating γ to β ratios was found.

Loss of β-Globin Preferential Expression With the p2γβE Construct

Preferential expression of the human β-globin gene in MEL cells was not found in an earlier study using a construct similar to p432γβE but lacking the HS4 and HS3 elements [11]. Constructs lacking HS4 (p32γβE) or HS4 and 3 (p2γβE) were prepared (Fig. 1C) to investigate whether the preferential β-globin gene expression observed in our study was due to inclusion of additional LCR sequences.

The γ to β mRNA ratio for MEL cells containing stably-integrated p32γβE was 0.37 (SD = 0.17 n = 5). The γ to β ratio for MEL cells containing stably-integrated p2γβE was 1.67 (SD = 0.92, n = 5) (Fig. 3A, Table I) which is significantly higher than the γ to β ratio found with p32γβE ($P < 0.01$) or p432γβE ($P < 0.005$). Inclusion of both HS2 and HS3 in the linked γ- and β-globin gene construct was sufficient for obtaining preferential expression of the human β-globin gene in MEL cells. However, inclusion of only HS2 in the constructs resulted in increased γ expression.

Partial Repression of the γ-Globin Gene by HS3

The difference in γ to β ratio between p2γβE and p32γβE could be due to changes in γ-globin gene expression, β-globin gene expression, or both. Expression levels of human globin genes were determined relative to endogenous mouse β-globin and normalized for gene copy number. The globin gene copy numbers were determined using multiplex PCR on the different pools of stably-transfected MEL cells. The mean numbers of human β-globin genes per cell were assumed to be equivalent to the γ-globin gene copy number in linked constructs. The average human globin mRNA levels on a per gene copy basis was calculated using PCR-determined gene copy per cell and results of the RNase assay for human and mouse β-globin mRNA. The human β-globin mRNA was quantitated as a per cent of total mouse β-globin mRNA (see Fig. 3B and Table I for representative assays and values). The mean β-globin gene expression was 30% of endogenous mouse β-globin gene expression on a per gene copy basis for MEL pools stably-transfected with p432γβE. The analogous values for the γ-globin genes were calculated using the β-globin gene values and the results of the γ to β mRNA ratios; the mean γ-globin gene expression was 13%.

The mean expression per human globin gene copy was also determined for the MEL cells stably-transfected with the p32γβE and p2γβE constructs. For the p32γβE constructs, mean expression was 9% and 26% for the γ- and β-globin genes, respectively similar to the corresponding

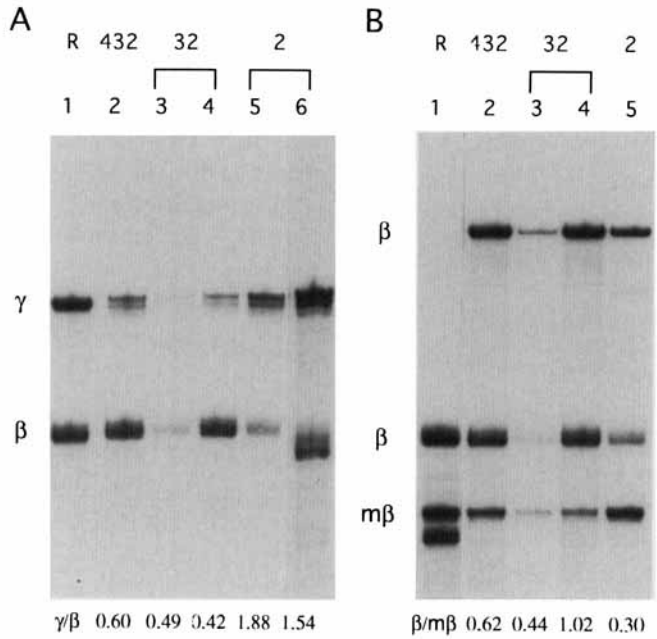

Fig. 3. The γ to β mRNA ratios in stably-transfected MEL cells indicate preferential expression of the β-globin gene from the p32γβE construct but not from the p2γβE construct. A: Representative results from RNase protection assays of γ and β mRNA levels from independent pools of MEL cells stably-transfected with p432γβE, p32γβE, or p2γβE. The γ to β mRNA ratio for each representative sample shown is given below. B: RNase protection assay of human β and mouse β mRNA levels from MEL pools (and reticulocyte control) shown in A (lanes 1–5). R, reticulocyte controls mixed with MEL RNA; the band just below "mβ" in lane 1 is analogous to the upper β band present in the transfected modified β mRNA (see Methods). Mean γ to β mRNA ratios are listed in Table I. The human β to mouse β ratio for each representative sample is shown below.

TABLE I. Comparison of Human Globin Gene mRNA Levels and Gene Copy Numbers in Stably-Transfected MEL Cells for p432γβE and Related Constructs*

Construct	γ/β mRNA	Human globin gene copy number/cell		mRNA level/ gene copy/cell (%)		Corr. γ/β
		γ	β	γ	β	
p432γβE (n = 8)	0.52 ± 0.17	13 ± 10		13 ± 6.9	30 ± 23	
p32γβE (n = 5)	0.37 ± 0.17	20 ± 14		9.4 ± 7.9	26 ± 19	
p2γβE (n = 5)	1.67 ± 0.92	10.5 ± 8.5		55 ± 36	34 ± 15.5	
p432γ + p432βE (n = 7)	0.80 ± 0.31	5.6 ± 2.9	8.5 ± 6.3	25 ± 14	27 ± 24	1.14 ± 0.65

*Mean values (± SD) are given for pools of MEL cells stably-transfected with the indicated construct. Levels of mRNA/gene copy/cell are given as % of mouse β-globin mRNA level/gene copy/cell. In the MEL line used (745), β^{maj} mRNA levels ≅ β^{min} mRNA levels [37]. Therefore, 4 mouse β gene copies/cell were used for these calculations. For linked constructs, β copy numbers equal γ copy numbers. Unlinked construct γ/β ratios were corrected based on individually determined γ and β gene copy numbers.

values (13%, 30%) for p432γβE (Table I, Fig. 4). By contrast, the mean expression was 55% and 34% for the γ- and β-globin genes, respectively, for p2γβE (Fig. 4). Thus, a 4- to 5-fold increase in γ expression was seen with p2γβE relative to γ expression from p32γβE or p432γβE. The mean gene copy number for p2γβE (10 copies/cell) was comparable to p432γβE (13 copies/cell) and unlikely to influence these results. Indeed, the same finding is made when only using data from pools found to contain 10 copies or less. The decrease in γ-globin gene expression from p32γβE and p432γβE suggests that HS3 can partially repress expression of the γ-globin gene in p32γβE and, by inference, p432γβE.

γ- and β-Globin Genes Transfected on Separate Plasmids

Plasmids containing either a γ- or a β-globin gene linked to a core LCR were cotransfected into MEL cells

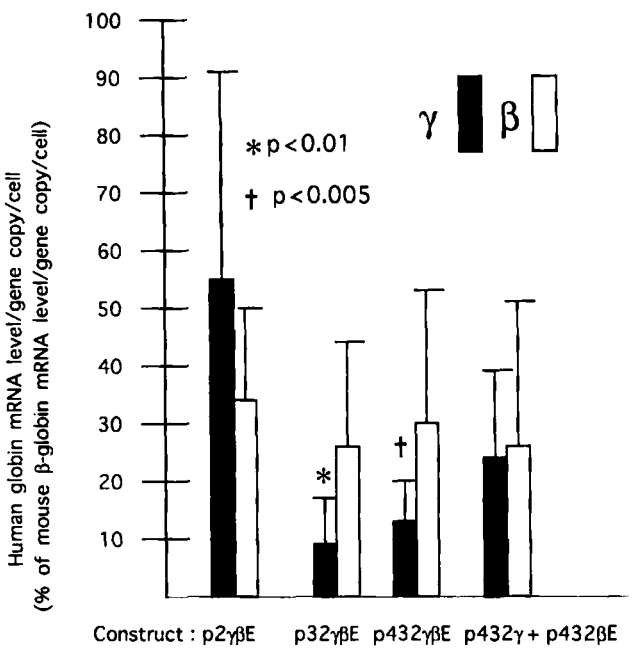

Fig. 4. Partial repression of γ- but not β-globin gene expression in stably-transfected MEL cells by inclusion of LCR HS3. Expression of human γ- and β-globin genes is presented as mRNA levels relative to endogenous mouse β-globin mRNA levels normalized per gene copy as listed in Table I. Each bar represents the mean value (with standard deviation) for all pools analyzed for the different constructs used. Significant *P* values shown for γ expression are relative to the p2γβE construct.

to determine whether the expression pattern observed with the linked globin gene constructs would also be found with unlinked globin gene constructs. If competition of the two globin genes for the single LCR contributes to the repression of the γ-globin gene, then greater γ expression would be observed with unlinked globin genes each with their own LCR.

The unlinked pair of constructs used was p432γ + p432βE in stable transfected experiments. The γ to β mRNA ratio was higher for this pair of unlinked constructs (1.14) than the comparable linked construct, p432γβE (0.52) (Table I) and is indicative of equivalent expression of the γ- and β-globin genes. The γ to β ratio for the unlinked constructs was adjusted for the γ- and β-globin gene copy numbers since they were not on the same plasmid. The PCR-determined gene copy numbers were used to adjust the γ to β ratios determined from the RNAse assay. The higher ratio for unlinked transfected constructs suggest competition of the two globin genes for the single LCR occurs in the linked constructs.

Gene copy numbers were also used to determine the mean expression per human globin gene copy per cell. The mean unlinked γ-globin gene expression per gene copy was 25% of mouse β-globin (Fig. 4, Table I). This value is about midway between the expression level seen in the corresponding linked constructs containing HS4 and 3 (13%) and the p2γβE construct (55%). Expression of the unlinked human β-globin genes was 27% slightly lower than the level for the p432γβE (30%) and the p2γβE (34%) constructs. The higher γ-globin gene expression from the unlinked transfected constructs suggests that competition for LCR activation contributes to some but may not account for all of the γ-globin gene repression.

DISCUSSION

Expression levels of the transfected globin genes on a per copy basis showed some variability but significant expression of both globin genes was found for all the MEL pools analyzed. For example, in the p432γβE-stably-transfected MEL pools, γ-globin gene expression ranged from 6 to 25% of mouse β, while human β-globin gene expression ranged from 10 to 82% of mouse β. The range of expression for the other constructs was similar. The total (γ + β) mean expression for p432γβE-stably-transfected MEL pools was 43% (Table I) comparable to that (52%) of p432γ + p432βE-stably-transfected MEL pools. Thus the change in γ to β ratios for the linked constructs is the result of increased β-globin gene expression with a concomitant reduction in γ-globin gene expression.

The highest γ-globin gene expression was obtained with the p2γβE-stably-transfected MEL pools (66%) in which the β-globin gene expression was comparable (35%, Table I) to the HS432 linked constructs (e.g., p432γβE). The gene copy number for p2γβE (11 copies/cell) was also comparable. Thus, inclusion of HS3 (and HS4) appears to reduce linked γ-globin gene expression. The γ- to β-globin mRNA ratio for the transfected p32γβE was 0.37 which is equivalent to the γ-globin gene contributing 27% of the total transfected globin gene expression (γ/γ + β). The transfected γ-globin gene cannot be considered normally regulated as the normal in vivo level of γ-globin gene expression is 1% or less in human adults.

Competitive interaction of γ- and β-globin genes for LCR activation in stably-transfected MEL cells was not found in earlier reports [11,30]. Morley et al. did not find competition using a construct, HS2Aγβ, similar to p2γβE used in our study [11]. p2γβE did not exhibit competition in our study due to the lack of additional LCR elements. Balta et al. also did not find evidence for competition using a construct containing all four LCR HS elements linked to γ- and β-globin genes [30]. Their analysis may be affected by the Aγ enhancer which had a positive effect on γ-globin transgene expression in the MEL 179 cell line they used despite the negative role they argue for the Aγ enhancer in human adult cells. We found no effect of the Aγ enhancer in the MEL 745 line. The

difference in results is, therefore, likely due to the details of the construct or the variant MEL cell line used.

γ- to β-globin switching occurs in transgenic mice with just the β-globin gene cluster excluding the LCR region; however expression is low and variable [31]. The strong enhancing activity of the LCR is required for consistent high level expression of the globin genes but must be guided somehow to act on those globin genes appropriate to the developmental stage. Our data provides evidence for different parts of the LCR having different roles in such mechanisms. The inclusion of HS3 core sequences reducing γ-globin expression but not the linked β-globin gene indicates that the enhancer activity of HS2 can be selectively blunted on γ-globin genes. Engel has proposed a model in which each LCR element acts upon a separate globin gene [32]. The HS3 element is most likely to associate with γ-globin genes based on the preferential enhancement of γ-globin genes seen in transgenic mice when HS3 alone is linked to γ- and β-globin genes [10,17]. The 225 bp HS3 core region defined by Grosveld and colleagues [24] and used in this study contains only CACC and GATA binding sites but not the upstream NF-E2 site [33]. We speculate that transcription factors (such as Sp1) binding at the CACC sites in HS3 interact with the γ-globin promoter at the expense of interacting with HS2. The transcription factor, EKLF, has also been described as binding to CACC sites but binds strongly to CACC in the β-globin promoter and poorly to the γ-globin promoter or the LCR [34]. This difference in CACC site preference has been proposed as a mechanism for fine tuning the enhancing properties of the LCR away from γ-globin genes and to β-globin genes in adults [35]. Our results suggest that this "fine-tuning mechanism" may involve selective sequestration of γ-globin promoter elements away from the enhancer activity in HS2 managed by the differential site preferences of factors. Precedent for enhancer sequestration to regulate promoter usage has been described in the muscle aldolase A gene system [36].

CONCLUSIONS

These studies clearly show that preferential expression of a transfected β-globin gene over a linked γ-globin gene can be obtained in MEL cells if the transfected globin genes are also linked to an LCR containing more than HS2. One critical component of preferential activation by the LCR of the β-globin gene is the presence of hypersensitive sites in addition to HS2. Comparison of transgene expression levels from constructs p32γβE and p2γβE indicates partial repression of γ globin gene expression by inclusion of the 225 bp HS LCR element. Repression of γ-globin gene expression occurred in part as a result of the β-globin gene competing better for activation by the LCR as equal expression of the γ- and β-globin genes was obtained if the genes were not linked on the same plasmid to a single LCR. The competitive advantage of the transfected β-globin gene is not likely to depend on chromatin structure because it was observed in both transient and stable transfection experiments.

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